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Heterologous overproduction of β-fructofuranosidase from yeast *Xanthophyllomyces dendrorhous*, an enzyme producing prebiotic sugars

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Abstract The β-fructofuranosidase Xd-INV from the yeast *Xanthophyllomyces dendrorhous* is the largest microbial enzyme producing neo-fructooligosaccharides (neo-FOS) known to date. It mainly synthesizes neokestose and neonystose, oligosaccharides with potentially improved prebiotic properties. The *Xd-INV* gene comprises an open reading frame of 1995 bp, which encodes a 665-amino acid protein. Initial N-terminal sequencing of Xd-INV pointed to a majority extracellular protein of 595 amino acids lacking the first 70 residues (potential signal peptide). Functionality of the last 1785 bp of *Xd-INV* gene was previously proved in *Saccharomyces cerevisiae* but only weak β-fructofuranosidase activity was quantified. In this study, different strategies to improve this enzyme level in a heterologous system have been used. Curiously, best results were obtained by increasing the protein N-terminus sequence in 39 amino acids, protein of 634 residues. The higher β-fructofuranosidase activity detected in this study, about 15 U/mL, was obtained using *Pichia pastoris* and represents an improvement of about 1500 times the level previously obtained in a heterologous organism and doubles the best level of activity obtained by the natural producer. Heterologously expressed protein was purified and characterized biochemically and kinetically. Except by its glycosylation degree (10 % lower) and thermal stability (4–5 °C lower in the 60–85 °C range), the properties of the heterologous enzyme, including ability to produce neo-FOS, remained unchanged. Interestingly, besides the neo-FOS referred before blastose was also detected (8–22 g/L) in the reaction mixtures, making Xd-INV the first yeast enzyme producing this non-conventional disaccharide reported to date.

Keywords Extracellular β-fructofuranosidase · *Xanthophyllomyces dendrorhous* · Neo-fructooligosaccharides · Heterologous expression · *Pichia pastoris* · Blastose

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Introduction

In the last couple of decades, the gut microbial communities have aroused huge interest. It is well known that the intestinal microbiota plays an important role in the immune system, which has even led to a new physiological classification of a human being (Arumugam et al. 2011). Fructooligosaccharides (FOS) selectively stimulate the growth and activity of health-promoting Lactobacilli and Bifidobacteria, a so-called prebiotic effect (Swennen et al. 2006) and contribute to the prevention of cardiovascular diseases, colon cancer, and osteoporosis (Kaur and Gupta 2002).

β-Fructofuranosidases (invertases; EC 3.2.1.26) catalyze the release of β-fructose from nonreducing termini of various β-D-fructofuranoside substrates, but also may transfer such fructose units to other molecules favoring the synthesis of a broad array of glyco-compounds. In particular, the microbial enzymes synthesize FOS, in which one to three fructosyl moieties are linked to one sucrose molecule (Sangeetha et al. 2005). Different types of FOS series can be distinguished: ^{1}F-FOS, containing β-(2-1)-linked fructose units (with an inulin-type structure; e.g. 1-kestose or nystose), ^{6}F-FOS, containing β-(2-6)-linked fructose units (with a levan-type structure; e.g. 6-kestose), and ^{6}G-FOS where the β-(2-6) link connects fructose and the glucosyl moiety of sucrose (neo-FOS; e.g. neokestose or neonystose). Among the prebiotic oligosaccharides, β-(2–6)-linked FOS possesses enhanced properties and

chemical stability than the traditionally commercialized β-(2–1) FOS typically produced by the *Aspergillus* species (Kilian et al. 2002; Lim et al. 2007; Sabater-Molina et al. 2011). These sugars are usually synthesized by acid hydrolysis of levans polymers, albeit through a non-environmental friendly process (Bekers et al. 2002). Alternatively, their enzymatic synthesis can be achieved with enzymes from yeasts. For instance, the β-fructofuranosidases from *Saccharomyces cerevisiae* (Farine et al. 2001), *Schwanniomyces occidentalis* (Alvaro-Benito et al. 2007; 2010a), and *Rhodotorula dairenensis* (Gutierrez-Alonso et al. 2009) produce mainly 6-kestose, whereas the enzyme from the basidiomycete *Xanthophyllomyces dendrorhous* (formerly *Phaffia rhodozyma*) synthesize neokestose (Chen et al. 2011; Linde et al. 2009).

The *X. dendrorhous* yeast produces astaxanthin, a red-orange pigment with antioxidant properties. Industrial interest of this carotenoid has led to intensify the molecular and genetic study of this yeast. As a result, several genes involved in the astaxanthin biosynthetic pathway have been characterized, as well as some others including those of the endo-β-1,3 glucanase, β-amylase, α-glucosidase, or the referred Xd-INV among others (see in Linde et al. 2009). Natural function of β-fructofuranosidase Xd-INV is still unknown but very plausibly could be involved in providing a carbon source for the yeast. This enzyme has been characterized biochemically and kinetically; it is a 160–200-kDa glycoprotein that displays maximum activity (hydrolase and transferase) at 60–70 °C. The neo-FOS levels obtained by this protein (>100 g/L) are the largest reported for any microbial enzyme as far as we know (Linde et al. 2012). The *Xd-INV* gene (1995 bp corresponding to a 665-amino acid) was previously expressed in *S. cerevisiae* but only a weak β-fructofuranosidase cellular activity (<10 mU/mL yeast culture) was quantified in this heterologous system (Linde et al. 2009).

This work describes the strategies used to try to increase the Xd-INV levels in different heterologous expression systems, as well as the purification and characterization of the enzyme expressed in our best option, increasing the protein N-terminus sequence in 39 residues and *Pichia pastoris*. For practical interests, the protein expressed in this system has several advantages over that expressed in the natural producer, including large-scale production and being in an excellent genetic background for further functional improvement by bioengineering.

Materials and methods

Strains, growth, and expression media

X. dendrorhous ATCC MYA-131 was grown in MMM (yeast nitrogen base w/o amino acids [YNB] 0.7 %, maltose 2 %; all *w*/*v*) as referred previously (Linde et al. 2009). *S. cerevisiae* EUROSCARF Y02321 (BY4741; MAT a; *his3Δ1*; *leu2Δ0*; *met15Δ0*; *ura3Δ0*; YIL162w::kanMX4) and *P. pastoris* GS115 (*his4*$^{-}$) (Invitrogen, Carlsbad, CA, USA) were used as expression hosts. *S. cerevisiae* was grown in medium YED (yeast extract 1 %, peptone 1 % glucose 2 %; all *w*/*v*); for selection and protein induction, SC was used (YNB 0.67 %, leu 0.1 %, his and met 0.05 %; all *w*/*v*) with glucose 2 % (SCD) or galactose 2 % (SCGal), respectively. *P. pastoris* was cultured in YED; transformants carrying constructions based on plasmids pIB and pGAPZαA were selected on MD (YNB 1.34 %, biotin 4×10^{-5} %, glucose 2 %; all *w*/*v*) and YED + Zeocin (YED but peptone 2 % and 100 μg/mL Zeocin) media, respectively. Expression of proteins in *P. pastoris* was analyzed using MMD (YNB 0.7 %, glucose 0.5 %; for pGAPZαA derived constructions), MD (for QDNS-pIB2 construction) and BMM after growing in BMG (both media same as MD but in potassium phosphate pH 6.0 and methanol 0.5 % or glycerol 1 % as carbon source, respectively; for QDNS-pIB4 constructions). Growth was monitored spectrophotometrically at a wavelength of 600 nm (A_{600}). The *E. coli* DH5α strain was used as a host for DNA manipulations using the standard techniques.

DNA amplification, cloning, and transformation

β-Fructofuranosidase *Xd-INV* gene from *X. dendrorhous* (GenBank accession no. FJ539193.2) comprises an open reading frame (ORF) of 1995 bp (1998 bp including the STOP triplet) corresponding to a 665-amino acid polypeptide. Two fragments from *Xd-INV* lacking the first 93 or 210 bp and responsible for the synthesis of Xd-INV polypeptides beginning with the amino acid sequence QDNS or FIAP, respectively, were amplified by PCR from a cDNA library (Linde et al. 2009). Primers QDNS*EcoR*I/INV*Not*I and INV*EcoR*I/INV*Not*I (Table 1) were used to obtain the 1902 and 1785 bp fragments responsible for the synthesis of 634 and 595 amino acids polypeptides, respectively. Both fragments were included into plasmid pGAPZαA (Invitrogen) fused to the 267-bp fragment of the *S. cerevisiae* MFα1 secretion signal sequence that includes the ATG initiation codon. The constructions QDNS-pGAP and INV-pGAP thus generated were used as template to amplify *Xd-INV* fragments of 1902 and 1785 bp fused to the MFα signal peptide sequence. Primers MF*Kpn*I/INV*Not*I were used to amplify the fusion products that were subsequently included in plasmid pYES2 (Invitrogen) generating constructions QDNS-pYES2 and INV-pYES2 (Table 2). Finally, primers MF*Kpn*I/pIB*Pst*I were used to amplify the 1902-bp fragment fused to the MFα signal peptide sequence that was included in plasmids pIB2 and pIB4 (Sears et al. 1998) generating the constructions QDNS-pIB2 and QDNS-pIB4. Targets for specific restriction enzymes were included in the primers to allow inclusion of amplification products in

Table 1 Primers used in this study

Primer	Sequence
QDNS*EcoR*I	ACGAATTCCAAGATAACTCCACCTCGTCCTCC
INV*EcoR*I	AAGAATTCTTCATTGCACCTGAAGGCTGCAT
INV*Not*I	AGTAGCGGCCGCTTAGTAACCAGCAAAGAGAC CG
MF*Kpn*I	GGGGTACCATGAGATTTCCTTCAATTTTTACTGC TGTT
pIB*Pst*I	GGCCTGCAGTTAGTAACCAGCAAAGAGACCG TAC
MA012F	CCTTCCCTGTTGAGGGTTAC
MA011R	CCGACTCGTTGAGCATCAC

Restriction sites are underlined

the specific plasmids. Integrity of *Xd-INV* in all constructions was verified by DNA sequencing.

Construction based on pYES2 was included in *S. cerevisiae* by the lithium acetate method (Alvaro-Benito et al. 2010a). Constructions based on pGAPZαA, pIB2, and pIB4 (5–10 μg) were linearized with *Avr*II for pGAPZαA and *Stu*I for pIB vectors, respectively, and transformed into *P. pastoris* by electroporation according to the manual for protein expression in *Pichia* (Invitrogen). Inclusion of *Xd-INV* gene in the genomic DNA transformants was confirmed by PCR using the primers MA012F and MA011R (Table 1), both directed to internal *Xd-INV* sequences, which generate a 318-bp amplification product. Transformants including the empty vectors pYES2, pGAPZαA, pIB2, and pIB4 were also obtained and used as controls.

Expression, purification, quantification, and identification of the β-fructofuranosidase Xd-INV

Expression of *Xd-INV* in *S. cerevisiae* and *P. pastoris* was analyzed using the media referred in Table 2. The optimal conditions were defined in a time course, and the heterologous activities were evaluated by measuring the β-fructofuranosidase activity (sucrose hydrolysis) in culture filtrates and soluble cellular fractions after cell lysate using Yeast Buster™ (Novagen, San Diego, CA, USA).

Table 2 Xd-INV activity detected in heterologous yeasts

Construction	Host/promoter	Activity (mU)	Expression condition
INV-pYES2	*S. c./pGAL1*	2	SCGal/24 h
INV-pGAP	*P. pastoris/pGAP*	16	MMD/72 h
QDNS-pYES	*S. c./pGAL1*	100	SCGal/24 h
QDNS-pGAP	*P. pastoris/pGAP*	900	MMD/72 h
QDNS-pIB2	*P. pastoris/pGAP*	5000	MD/24 h
QDNS-pIB4	*P. pastoris/AOX1*	14700	BMM/24 h

Extracellular activity/mL detected in yeasts carrying the indicated construction are showed. Standard error was lower than 15 %

S.c., S. cerevisiae

For purification of β-fructofuranosidase activity expressed in *P. pastoris*, transformants carrying the construction QDNS-pIB4 were grown in 25 mL of BMG during 24 h and then in 200 mL of BMM for 24–36 h. Cells were removed at 6000×*g* for 15 min. Extracellular fraction (about 15 U of β-fructofuranosidase activity/mL of culture) was concentrated (about 12-fold) and fractionated trough 50000 MWCO, PES membrane by using a Vivaflow 50 system (Sartorius, Goettingen, Germany). The active fraction (16 mL; ~146 U/mL) was dialyzed in 20 mM HCl-Tris pH 7 (buffer A) and applied to a DEAE-Sephacel chromatography column (10 mL) equilibrated with buffer A. The proteins were eluted with a discontinuous gradient of 0, 0.05, 0.1, and 0.2 M NaCl (10 column volumes for each salt concentration) at flow rate of 1 mL/min. Active fractions (2 mL) eluted at 0.1 M NaCl were pooled (20 mL; 72 U/mL), dialyzed in buffer A, and concentrated using the Microcon YM-10 (Millipore, Cork, Ireland) system (0.7 mL; 1835 U/mL). ProtoBlue Safe Colloidal Coomassie stained (National Scientific, Atlanta, GA, USA)-sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE; 8 % polyacrylamide) of the samples confirmed the purity of the protein fractions. Precision Plus protein standards unstained 10–250 kDa (Bio-Rad, Hercules, CA, USA) were used as weight markers. Protein concentration was determined using the Bio-Rad microprotein determination assay and bovine serum albumin as standard. Peptide-N-glycosidase F (PNGase F; Sigma-Aldrich, St. Louis, MO, USA) treatment was performed according to the manufacter's protocol. β-Fructofuranosidase purified from *X. dendrorhous* as previously referred (Linde et al. 2012) was used as control.

The polyclonal invertase antiserum used for Western blotting was generated by injecting rabbits with commercially available *S. cerevisiae* invertase as previously referred (Alvaro-Benito et al. 2010b) and was used at a dilution of 1:6000. For immunoblots, extracellular proteins were prepared from *P. pastoris* cultures growing at 3–4 A_{600}, which were then concentrated and partly fractionated by filtration through Microcon YM-10 membranes. The proteins were resolved electrophoretically on polyacrylamide gel and transferred to Inmobilon-PVDF membranes (Bio-Rad). Binding of the antibody was detected using a secondary goat anti-rabbit IgG coupled to horseradish peroxidase (Dako, Glostrup, Denmark; 1:5000 dilution) and ECL Western blotting detection reagents (GE Healthcare Science Life, UK). Prestained protein standards 7–175 kDa (New England Biolabs, Ipswich, UK) were used as weight markers.

For protein mass spectrometry analysis, samples were excised from SDS-PAGE gel, digested with trypsin, desalted, and analyzed by matrix-assisted laser desorption ionization-

time-of-flight-mass spectrometry (MALDI-TOF) at the Proteomic Service of the Centro de Biología Molecular Severo Ochoa (Madrid).

Enzyme and kinetic analysis

Unless otherwise indicated, β-fructofuranosidase hydrolytic activity was determined by the dinitrosalicylic acid (DNS) method adapted to a 96-well microplate scale as described elsewhere (Alvaro-Benito et al. 2010a). The reaction mixture (50 μL) contained sucrose 2 % (*w*/*v*) in 0.1 M sodium phosphate buffer (pH 5.5), and the enzyme solution (5 μL) conveniently diluted to fit into a calibration curve was incubated at 60 °C for 10–20 min. One unit of β-fructofuranosidase activity was defined as that corresponding to the release of 1 μmol of reducing sugar per minute. The Michaelis-Menten kinetic constants were determined using 0–60 mM sucrose and 0–20 mM 1-kestose. The plotting and analysis of the curves was carried out using SigmaPlot software (version 11.0), and the kinetic parameters were calculated fitting the initial rate values to the Michaelis-Menten equation.

The estimation of hydrolase activity at different pH values (4.0–9.0) and temperatures (25–90 °C) was carried out under the aforementioned conditions using sucrose as substrate and 60 °C and pH 5.5, respectively. The buffers used were citric acid-sodium citrate (pH 4.0–5.0), Na_2HPO_4-NaH_2PO_4 (pH 5.0–7.5), and Tris-HCl (7.5–9.0), all at 100 mM. The thermostability refers to the temperature required for 50 % enzyme inactivation (enzyme half-life) after heating 0.3–0.5 U of the pure enzyme at different temperatures (60–80 °C) during 30–120 min and was determined by removing samples at regular intervals and estimating the residual β-fructofuranosidase activity. β-Fructofuranosidase Xd-INV activity purified from *X. dendrorhous* as referred in Linde et al. (2012) was used as control in all these tests. All the reactions were performed in triplicate.

Xd-INV activity was detected by zymogram analysis using non-denaturing gradient gels (4–15 %; Bio-Rad) stained with 2,3,5-triphenyltetrazolium chloride (Linde et al. 2009). Invertase from *S. cerevisiae* (Novozymes, Bagsvaerd, Denmark) and Xd-INV purified from *X. dendrorhous* were used as controls. Bovine serum albumin (Sigma-Aldrich) was used as weight marker.

Batch production of fructooligosaccharides

The transferase activity was tested in duplicate using a sucrose solution of 600 g/L in 0.1 M sodium acetate buffer pH 5.5. Total reaction volume was 1–1.5 mL, and final activity in the mixture was adjusted to 0.5–1.0 U/mL. The mixture was incubated at 60 °C in an orbital shaker Vortemp 56 (Labnet International, Edison, NJ, USA) at 100 rpm. At different times, 0.1 mL aliquots were extracted, treated for 10 min at 100 °C to inactivate the enzyme, and diluted with water (1:15). Samples were filtrated through 0.45 μm cellulose centrifuge filters (National Scientific) and analyzed by High performance liquid chromatography using a quaternary pump (Delta 600E, Waters, Cerdanyola del Vallès, Barcelona, Spain) coupled to a 5-μm Kromasil-NH_2 100A column (4.6×250 mm; Análisis vínicos, Tomelloso, Toledo, Spain). Detection was performed using a light-scattering detector (Sedex 75, Sedere, France) equilibrated at 54 °C. Acetonitrile/water 82:18 (*v*/*v*), degassed with helium, was used as the mobile phase at 1 mL/min for 25 min. Glucose, fructose, sucrose, and 1-kestose were used as standards. Results were analyzed using the Millennium software (Waters). HPLC analysis of each sample was performed in duplicate.

Results

Cloning and heterologous expression of the β-fructofuranosidase Xd-INV from *Xanthophyllomyces dendrorhous*

The open reading frame of the β-fructofuranosidase *Xd-INV* gene from yeast *X. dendrorhous* consists of 1995 bp, which encodes a 665-amino acid protein with no apparent signal peptide sequence. Initial N-terminal sequencing of the extracellular Xd-INV protein showed the sequence FIAPEGWMNDPMGL (Linde et al. 2009), pointed to a majority extracellular protein lacking the first 70 amino acids (potential signal peptide). Functionality of the last 1785 bp of *Xd-INV* sequence (responsible for the protein synthesis without the potential signal peptide) was proved in *S. cerevisiae* using plasmid pVT103-L and the MFα1 spacer region (KREAEA) that would direct the encoded 595 amino acid protein secretion into the yeast extracellular medium. However, only weak β-fructofuranosidase activity (about 10 mU/mL) was quantified in the host yeast cells, and no extracellular activity was detected (Linde et al. 2009). By replacing MFα1 spacer region by the α factor, signal peptide (89 amino acid MFα1) activity was detected in the yeasts extracellular media, indicating that the largest signal peptide could direct the secretion of the enzyme, but unfortunately, Xd-INV activity levels were not improved (construction INV-pYES2 in Table 2). Activity level neither significantly increased by using *P. pastoris* as expression system (about 16 mU/mL; construction INV-pGAP in Table 2).

Analysis of the potential signal peptide from Xd-INV showed the sequence Arg30-Arg31-Gln32-Asp33-Asn34-Ser35 (RRQDNS), a potential target for proteases such as KEX2. Curiously, by increasing the N-terminus sequence of protein in 39 residues (peptide that starts with the QDNS sequence of 634 amino acids; constructions QDNS-pYES2

and QDNS-pGAP), β-fructofuranosidase activity improved by almost 50 times in the two used expression systems (Table 2). In addition, the activity levels detected in *P. pastoris* increased in about 5 and 16 times more by using construction derivatives from vectors pIB2 and pIB4 (constructions QDNS-pIB2 and-pIB4), in which this activity expression was controlled by the *GAP* and *AOX1* strong promoters (Vogl and Glieder 2013), respectively. The higher β-fructofuranosidase level detected in this work, about 15 U/mL culture, was obtained in *P. pastoris* transformants grown during 24 h for an extracellular medium pH of about 5.2 units (Fig. 1).

Characterization of the heterologous protein expressed in *P. pastoris*

P. pastoris cells transformed with QDNS-pIB4 construction and showing β-fructofuranosidase activity secreted basically only a major protein into the medium of about 130 kDa, which was absent in the control yeasts transformed with the empty vector pIB4 (Fig. 2a). Heterologous protein was purified to homogeneity by using basically a single DEAE-Sephacel chromatography step (Fig. 2a) and about 43 % of the β-fructofuranosidase activity was recovered (~757 U/mg protein). Molecular mass of the purified protein was at least 30 kDa lower than that of enzyme expressed in *X. dendrorhous* (160–200 kDa), and treatment with PNGase F resulted in a mass shift to about 66 kDa (Fig. 2b), the size of Xd-INV expressed in the natural producer after deglycosylation (Linde et al. 2009). Presuming that the glycosylated and unglycosylated forms behave similarly in the gel, N-linked oligosaccharides appear to represent ~50 % (~64 kDa) of the total heterologous protein mass vs. the ~60 % (~94 kDa) that represented in the protein produced by *X. dendrorhous*. Protein expressed in *P. pastoris* was recognized with anti-INV antibodies (Fig. 2c) and yielded a band of about 260 kDa in activity-staining gels (Fig. 2d) suggesting that the active enzyme was also likely to function as a dimmer. Finally, analysis by MALDI-TOF and fingerprinting indicated that the protein expressed in *P. pastoris* undoubtedly showed the amino acid sequence predicted by the *Xd-INV* gene sequence (18 tryptic peptides from Xd-INV were detected; data not shown). The heterologously expressed protein was named pXd-INV.

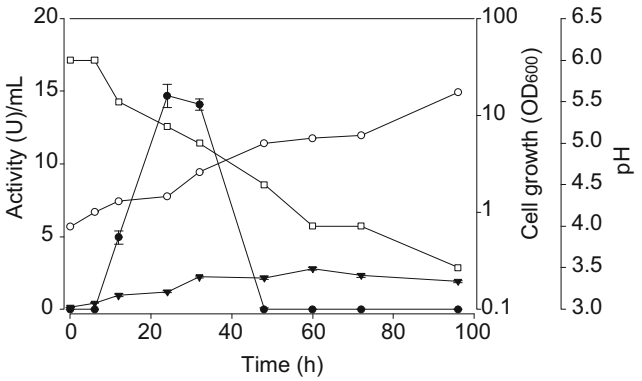

Fig. 1 Time course of pXd-INV production and extracellular pH change. Inocula from *P. pastoris* transformed with the QDNS-pIB4 construction were grown in a 1-L flask containing 200 mL of BMM (*empty circles*). Samples withdrawn at the indicated times were analyzed for pH (*empty squares*) as well as cellular (*full triangles*) and extracellular fructofuranosidase activity (*full circles*) using sucrose as substrate. Each point of activity represents the average of three independent measurements; standard error is indicated. Similar results were obtained for two other different yeast cultures (data not shown)

Biochemical characterization of pXd-INV hydrolytic activity

Since the glycosylation degree of the enzyme expressed in *P. pastoris* (pXd-INV) and *X. dendrorhous* (Xd-INV) was different, protein produced by the two yeast species was purified and their biochemical properties were compared. Regardless the producing yeast, enzyme displayed maximum activity at pH 5.0–6.6 and 60–70 °C, with about 90 % of activity, maintained in the range from 60 to 75 °C (Table 3). However, when it was incubated without substrate in the range from 60 to 85 °C for 30–120 min and then β-fructofuranosidase activity were assayed, Xd-INV and pXd-INV maintained 50 % of their activity (half-life) at 80–82 and 76–77 °C, respectively. Kinetic parameters of the enzyme expressed in the two systems were also slightly different when using sucrose and 1-kestose as substrates (Table 3), but showed similar catalytic efficiency (defined by the k_{cat}/K_m ratio) and hydrolyzed sucrose about 4 times more efficiently than 1-kestose regardless the producing yeast.

Transfructosylating activity of the β-fructofuranosidase expressed in *P. pastoris*

The production of FOS mediated by the enzyme expressed in *P. pastoris* and *X. dendrorhous* was analyzed at 60 °C under the conditions indicated in "Batch production of fructooligosaccharides". As expected, analysis of the reaction products generated similar chromatographic profiles (Fig. 3). Based on its chromatographic mobility and using standards previously purified as described (Linde et al. 2012; Zambelli et al. 2014), compounds corresponding to peaks 5 and 7 were identified as neokestose (Fru-(2–6)-Glc-(1–2)-Fruc) and neonystose (Fru-(2–6)-Glc-(1–2)-Fruc-(1–2)-Fruc), respectively (both included in series ^{6}G-FOS), peak 6 as 1-kestose (Glu-(1–2)-Fruc-(1–2) Fruc) (series ^{1}F-FOS), and peak 4 as blastose (Fru-(2–6)-Glc), a sucrose isomer member of the neo-FOS series. Proportion of oligosaccharides obtained in the biosynthetic reaction mixtures was very similar regardless the yeast producing the enzyme. Maximum FOS yield was reached for a conversion of 75–80 % (*w*/*w*) sucrose (Fig. 4). At this point, Xd-INV generated 30 % (*w*/*w*) FOS (180 g/L, of which 91 g/L correspond to neokestose, 71 g/L to 1-kestose and 18 g/L to

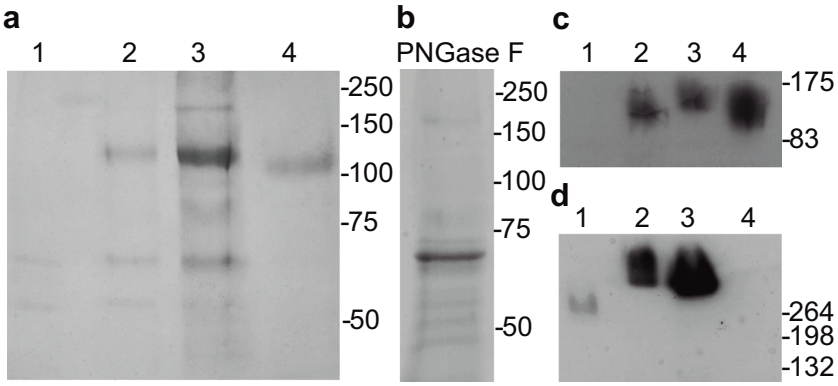

Fig. 2 PAGE, PNGase F, and Western analysis of Xd-INV expressed in *P. pastoris*. **a** Purification: the culture filtrate from *P. pastoris* expressing Xd-INV was subjected to SDS-PAGE before (*lane 2*) or after concentration using the Vivaflow 50 system (*lane 3*) and after DEAE-Sephacel column chromatography (*lane 4*). Culture filtrate from yeast transformed with the empty pIB4 plasmid was used as control (lane 1). **b** Concentrated culture filtrate expressing Xd-INV (panel **a** *lane 3*; 5 μ of total proteins) after digestion with 0.2 U of PNGase F for 90 min at 37 °C. **c** Western blot assay using anti-INV antibodies and culture filtrates from *P. pastoris* carrying pIB4 plasmid (*lane 1*), or QDNS-pIB4 construction (*lane 2*), and from *X. dendrorhous* expressing Xd-INV (*lane 3*). Invertase from *S. cerevisiae* was used as positive control (*lane 4*). **d** Xd-INV activity expressed in *P. pastoris* was revealed in situ (*lane 1*). Xd-INV purified from *X. dendrorhous* (*lane 2*) and invertase from *S. cerevisiae* (*lane 3*) were used as positive controls. *P. pastoris* including the empty pIB4 plasmid was used as negative control (*lane 4*). The *numbers on the right* indicate the positions of molecular mass standards in kDa

neonystose), 109 g/L of neo-FOS and pXd-INV 29 % (*w*/*w*) FOS (176 g/L, of which 95 g/L correspond to neokestose, 58 g/L to 1-kestose and 23 g/L to neonystose), and 118 g/L of neo-FOS. Blastose was obtained in both reactions, about 22 and 8 g/L by using Xd-INV and pXd-INV, respectively (Fig. 4a). To our knowledge, this is the first report describing the formation of blastose in a transfuctosylation reaction mediated by a yeast enzyme.

Discussion

The β-fructofuranosidase Xd-INV from *X. dendrorhous* is an extracellular glycoprotein that synthesizes neo-FOS (basically neokestose and neonystose), oligosaccharides with potentially improved prebiotic properties. To express this protein in a genetic background that allows its functional improvement is critical for biotechnological application. In this study, different strategies to successfully express this activity in heterologous yeasts have been used. Unpredictably, the best results were obtained by increasing the N-terminus sequence of the protein previously obtained by Edman sequencing (Linde et al. 2009) in 39 residues (protein of 634 amino acids vs. the previous of 595 amino acids). The higher β-fructofuranosidase activity detected in this work represents an activity improvement of about 1500 times the level previously obtained in a heterologous organism and doubles the best level of activity obtained by the natural producer of this protein in 70–100 h (~7 U/mL; Linde et al. 2009, 2012). Maximum activity level produced in *P. pastoris* was not maintained beyond 30 h of culture probably as consequence of intracellular proteases released to extracellular medium and/or activation of proteases acting at as the medium pH decreases, as has been referred by others who used this yeast as expression system (Huang et al. 2011; Lu et al. 2013). Really, even the lack of the first 70 residues initially detected by N-terminal sequencing of the protein produced in *X. dendrorhous* (Linde et al. 2009) could be caused by a proteolytic process where it could take part the aspartic

Table 3 Biochemical characteristics and kinetic parameters for pXd-INV and Xd-INV

	Biochemical characterization			Kinetic parameters			
	Optimum T (°C)	Thermostability (°C)	Optimum pH	Substrate	K_m (mM)	k_{cat} (s^{-1})	k_{cat}/K_m (s^{-1} mM^{-1})
pXd-INV	60–70	76–77	5.0–6.6	Sucrose	1.9±0.3	911±47	479±68
				1-Kestose	1.2±0.2	134±13	112±15
Xd-INV	60–70	80–82	5.0–6.6	Sucrose	3.9±0.8	1958±117	502±40
				1-Kestose	4.3±0.6	522±42	121±14

The reaction rate measurements were performed in triplicate. Values of k_{cat} were calculated considering a protein molecular mass of 66 kDa. Enzyme concentration, 9.3 μg/mL. The k_{cat}/K_m standard errors were obtained by fitting the normalized Michaelis-Menten equation as $v=(k_{cat}/K_m)[S]/(1+[S]/K_m)$

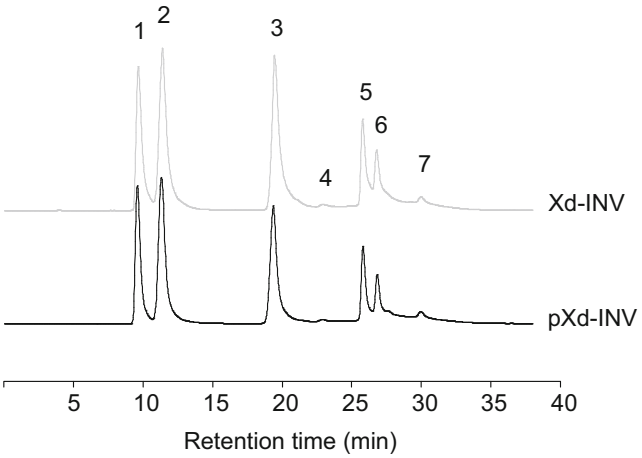

Fig. 3 HPLC chromatogram corresponding to the reaction of sucrose with β-fructofuranosidase. Chromatograms correspond to the reaction mixtures of the enzyme expressed in *X. dendrorhous* (Xd-INV; *gray line*) and *P. pastoris* (pXd-INV; *black line*) for about 75 % sucrose conversion. Peaks: *1*, fructose; *2*, glucose; *3*, sucrose; *4*, blastose; *5*, neokestose; *6*, 1-kestose; *7*, neonystose

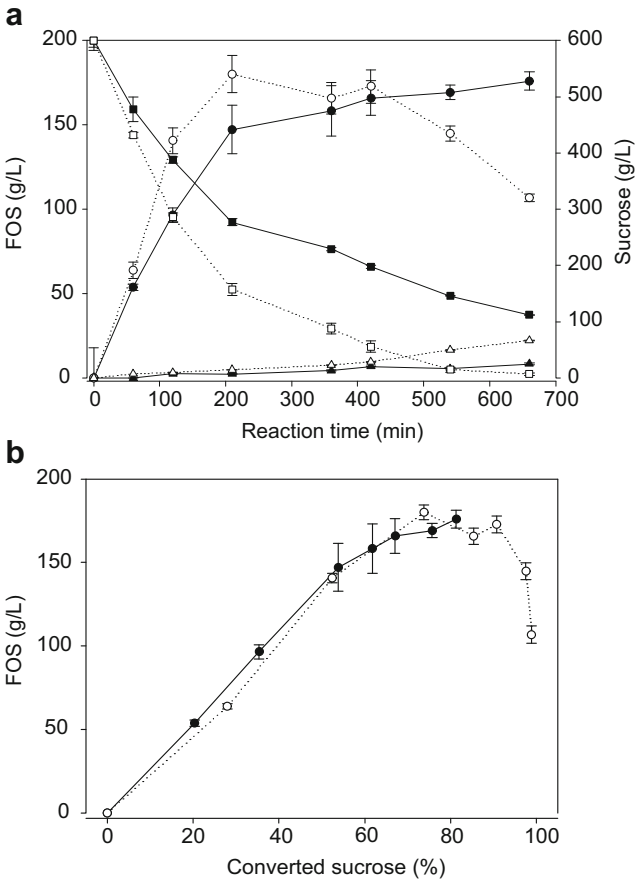

Fig. 4 Time course of FOS production catalyzed by the β-fructofuranosidase expressed in *X. dendrorhous* and *P. pastoris*. **a** Total FOS (*circles*), blastose (*triangles*), and sucrose (*squares*) quantified in the reaction mixtures. **b** FOS production vs. sucrose converted (%). Enzyme produced by *X. dendrorhous* (*empty symbols*) and *P. pastoris* (*full symbols*) were used. Standard error of data is indicated

protease of 36 kDa previously detected in this yeast (Bang et al. 1999). Indeed, traces of the Gln32-Asp33-Asn34-Ser35 sequence was also initially detected in the N-terminal sequencing of Xd-INV, indicating the presence (minority) of an extracellular protein lacking only the first 31-amino acid residues (data not shown). Clearly, the protein of 595 amino acids initially analyzed was truncated in the amino terminus, lacked of the first 39 residues, but even so it had some residual hydrolytic activity. To characterize the 3D structure of Xd-INV is essential to understand these data as well as the enzyme specificity, and currently, we are working in this direction.

Initially, *P. pastoris* was chosen as expression system primarily due to its inherent lack of β-fructofuranosidase (invertase) activity and high capacity for heterologous protein secretion (Tschopp et al. 1987; Vogl and Glieder, 2013). Further advantage of this yeast is the absence of α-1,3-linked mannosyl transferase, which produces the highly terminal α-1,3-linked mannosyl linkages in *S. cerevisiae*, and the less extensive hyper-mannosylation in *P. pastoris* compared to *S. cerevisiae* (Çelik and Çalik, 2012). No doubt N-glycosylation is one of the most common forms of protein post-translational modification in *P. pastoris*, where approximately 70–90 % Asn residues included in potential N-glycosylation sites (Asn-Xaa-Ser/Thr) are N-glycosylated. This process is species-specific (Piirainen et al. 2014) and often plays a crucial role in the secretion, stability, and/or activity of proteins expressed in *P. pastoris* (Han et al. 2014; Skropeta 2009). Thus, glycosylation degree was essential for secretion of the fungi *Volvariella volvacea* acetyl xylan esterase (Tian et al. 2012), thermal stability of the *Candida rugosa* lipase (Tang et al. 2001), catalytic activity and stability of the horseradish peroxidase (Capone et al. 2014), or the biochemical properties of β-glucuronidase GUS from *Penicillium purpurogenum* (Zou et al. 2013). In this context and since the glycosylation level of the protein here expressed in *P. pastoris* (pXd-INV) was lower than that of the one produced in *X. dendrorhous* (Xd-INV), biochemical properties of protein produced by the two yeast species were compared. Under the conditions used in this work, thermostability of the pXd-INV was reduced in about 4–5 °C (Table 3). Regardless the yeast producing the enzyme and except by its glycosylation degree and thermal stability, the characteristics of the heterologous enzyme do not differ substantially from that produced by *X. dendrorhous*. Enzyme always hydrolyzed sucrose 5–20 times more efficiently than β-fructofuranosidases produced by yeasts as *R. dairenensis* (90 s^{-1} mM^{-1}; Gutierrez-Alonso et al. 2009) or *S. occidentalis* (24 s^{-1} mM^{-1}; (Alvaro-Benito et al. 2010b).

Neokestose was the main transglycosylation product accumulated by whole *X. dendrorhous* cells from sucrose (Kritzinger et al. 2003) and also the major fructooligosaccharide (FOS) produced by β-fructofuranosidase Xd-INV (Linde et al. 2009, 2012) and pXd-INV. The transfructosylation

reactions always showed similar chromatographic profiles, with neo-FOS as major products, and the identification of blastose. This non-conventional disaccharide was probably produced by the neokestose hydrolysis because Xd-INV was unable to transfer the fructosyl moiety from sucrose to a new glucose unit (Gimeno-Perez et al. 2014). It is worth mentioning that to our knowledge, this is the first report describing the formation of blastose in a transfuctosylation reaction mediated by a yeast enzyme. The production of this small neo-FOS was initially described in submerged cultures of members of the fungal genus *Claviceps* (Flieger et al. 2005), later in reactions catalyzed by levansucrase from *Bacillus megaterium* (Homann et al. 2007), and recently from the fungal *Cladosporium cladosporioides* mycelium (Zambelli et al. 2014). The blastose amount obtained in this work, although slight, is significant considering that the largest production of this disaccharide reported to date was 34 g/L using the *C. cladosporioides* mycelium (Zambelli et al. 2014). Time course of FOS production catalyzed by pXd-INV was also slightly slower than that of Xd-INV, possibly due to its lower k_{cat} (Table 3). However, the production of FOS vs. the percentage of sucrose converted was almost identical for the enzyme expressed in the two systems (Fig. 4).

Altogether, our data show that overproduction of the β-fructofuranosidase from *X. dendrorhous* in *P. pastoris* was successful, without affecting its biotechnological utility for the synthesis of neo-FOS. This achievement is essential to carry out future mutational analysis of this enzyme in order to clarify the determinants of its specificity and to perform its operational improvement using molecular bioengineering techniques.

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Conflict of interests The authors declare that they have no conflict of interest.

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